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Biogenesis of ISP6, a Small Carboxyl-terminal Anchored Protein of the Receptor Complex of the Mitochondrial Outer Membrane*

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To study the biogenesis of ISP6, an outer membrane component of the mitochondrial protein translocation complex, two fusion proteins have been made by fusing ISP6 to either the carboxyl- or amino-terminal end of the mouse dihydrofolate reductase (DHFR). *In vitro* import experiments showed that when DHFR was placed at the carboxyl-terminal end of ISP6, the resulting fusion protein 6-DHFR inserted into mitochondrial membrane less efficiently than the other form of the fusion proteins. *In vivo* this fusion protein lost its ability to suppress the temperature-sensitive phenotype of an isp42 mutant, while the other fusion protein DHFR-6, which was found targeted correctly to mitochondria, suppressed the mutant as well as the wild-type ISP6. Further analysis showed that the binding and insertion of DHFR-6 to mitochondrial outer membrane was not affected by deletion of either of the two mitochondrial protein receptors or by the predigestion of mitochondrial surface proteins prior to import. Additional data indicated that ISP42, which closely associates with ISP6 in the translocation complex, does not likely play the role of a targeting partner for ISP6. In summary, these data suggest that ISP6 may target to mitochondria by sequences at its carboxyl terminus and that the import process of ISP6 is most likely distinct from that of most other mitochondrial precursors, which are recognized by protein receptors on mitochondrial surface.

The biogenesis of mitochondria depends on the synthesis and correct localization of a large number of proteins from the cytoplasm. This requires both the action of specific signals on the mitochondrial precursor proteins to direct them to their correct suborganellar location as well as the function of receptor complexes on the surface of the organelle (Schatz, 1993; Stuart *et al.*, 1993). Studies from different groups have now documented the presence of a dynamic receptor complex, which is required for the import of different classes of mitochondrial precursors (Pfanner *et al.*, 1992; Segui *et al.*, 1993). Both antibody-subfragment blocking studies and gene disruption experiments have shown that the receptor elements termed MAS70/MOM72 and MAS20/MOM19 define the key components of the receptor complex, which are responsible for the efficient import of essentially all mitochondrial precursors (Hines *et al.*, 1990; Sollner *et al.*, 1989, 1990; Moczko *et al.*, 1994). Although yeast strains that harbor simultaneous deletions of MAS70 and MAS20 fail to grow (Ramage *et al.*, 1993), recent studies show they can adapt and grow normally. This suggests that yeast may contain additional genes encoding potential receptor elements on the mitochondrial surface that may not be normally expressed (Lithgow *et al.*, 1994).

The parallel action of the MAS70 and MAS20 receptors in the receptor complex direct bound precursor proteins to a common translocation pore. This pore consists of the outer membrane protein ISP42, which has been shown by independent criteria to directly participate in the translocation of proteins across the membrane bilayer. Earlier studies from this laboratory have identified a small protein, ISP6, which is associated with ISP42 (Kassenbrock *et al.*, 1993). Early characterization of this protein has revealed that it is necessary for the function of temperature-sensitive alleles of ISP42. These studies are consistent with a function for ISP6 as one which is necessary to stabilize ISP42 so that either ISP42 is properly assembled into the translocation site or that the gating of ISP42 is assisted by this small protein (Hartmann *et al.*, 1994).

In the present study, we have examined the biogenesis of the ISP6 protein. This particular protein is very small. It is only 61 amino acids in length and anchors specifically to the mitochondrial outer membrane by a carboxyl-terminal anchor (Kassenbrock *et al.*, 1993). The delivery and interaction of small proteins into different intracellular membranes is of interest since several recently identified small carboxyl-terminal anchored membrane proteins are essential in biogenesis or function of membrane translocation complexes in endoplasmic reticulum and synaptic membranes (Kutay *et al.*, 1993; Dobberstein, 1994). In the case of the mitochondrial outer membrane, the identification of ISP6 in association with ISP42 provides an example of such a protein in association with an intracellular translocation complex. One noteworthy aspect of small carboxyl-terminal anchored proteins is that their intracellular targeting appears to be determined by the sequences either adjacent to or a part of the carboxyl-terminal anchor domain (Kutay *et al.*, 1993; Mitoma and Ito, 1992). If this is the case then, the mechanisms that operate for the targeting and localization of most other proteins do not appear to operate for the carboxyl-terminal anchored proteins. For example, the synthesis of these proteins must be essentially completed in order to make the signals available for localization. Thus, the delivery mechanism for these proteins likely utilizes other proteins after synthesis to assist their correct insertion and assembly. The ISP6 provides an interesting model for analysis of the components and mechanisms that operate for proper biogenesis of such a protein. In the present study, we have exploited the biochemical and genetic properties of ISP6 to define the basic features of its biogenesis.

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EXPERIMENTAL PROCEDURES

Strains—The *Saccharomyces cerevisiae* strains used in this study were w303/a (ade2–1 his3–11, 15 leu2–3, 12 trp1–1 ura3–1 can1–100), isp42–3 (Kassenbrock *et al.*, 1993), YTJB5 (a leu2–3, 112 ΔURA3 His4–519 mas20::URA3), and LEYL6 (a ade2–1 his3–11, 15 leu2–3, 12 trp1–1 ura3–1 can1–100 mas70::URA3). The *Escherichia coli* strain RR1 (supE44 hsdS20($r_B^-m_B^-$) ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1) was used to amplify plasmids.

Construction of Plasmids—Fusion constructs containing mouse dihydrofolate reductase and ISP6 sequence were prepared from plasmid pBS-3S1-Stu-*Bam*HI (Kassenbrock *et al.*, 1993) and pT7–2:DHFR[1] (Smagula and Douglas, 1988).

To construct DHFR-6, primers DHFR-N-H (CGC AAG ATC GAT TCT AGA A) and DHFR-C-B (CAC GGA TCC GTC TTT CTT CTC GTA GAC) were used to amplify a fragment from pT7–2:DHFR containing the DHFR gene by polymerase chain reaction while deleting the termination codon and introducing a *Bam*HI site at its 3′ end. This fragment was inserted between *Hin*dIII site and *Bam*HI site of plasmid pBlue-Script KS(−) to generate pBS-DHFR(ns). Another polymerase chain reaction was performed using primers 61-N-BAM (GCC GGA TCC AAA ATG GAC GGT ATG TTT) and M13 (−20) forward to introduce a *Bam*HI site just before first ATG of ISP6 gene from plasmid pBS-3S1-Stu-*Bam*HI. This second fragment was then inserted into the *Bam*HI site immediately after DHFR coding sequence in pBS-DHFR(ns). To transfer this fusion gene into pRS315gal, a derivative of pRS315 (Sikorski and Hieter, 1989), which contains a 685-base pair *Eco*RI-*Bam*HI fragment of GAL1 and GAL10 promoters (Johnston and Davis, 1984), the 1.2-kilobase pair fragment generated after digestion of pBS-DHFR-6 with *Xba*I was then inserted into *Xba*I site of pRS315gal with the orientation of 5′ end of DHFR adjacent to the GAL 1 promoter.

The strategy of constructing 6-DHFR was similar to above except primers 61-N-BAM/61-ORF (GCC GGG ATC CAA TTG TGG GGC CAA CAT) and DHFR-N-B (CAC GGA TCC CAT GGT TCG ACC ATT GAA C)/T7-LINK (GGC CAG TGT GAA TTC) were used to amplify fragments containing ISP6 and DHFR coding sequence, respectively.

In Vitro Transcription and Translation—Linearized plasmid DNA was transcribed and further translated *in vitro* as described (Cyr and Douglas, 1991).

Induction of Fusion Proteins and Subcellular Fractionation—Yeast transformations were performed by the lithium acetate method (Schiestl and Gietz, 1989). The yeast strains to be induced first grew overnight in yeast nitrogen base dextrose medium that contained 0.5% dextrose, and then they were diluted into yeast nitrogen base galactose medium that contained 2% galactose and harvested with an $A_{600\ nm}$ between 3 and 5. Subcellular fractionation was done as described (Hase *et al.*, 1984).

Binding and Import of Labeled Proteins—Labeled proteins were incubated with 50 μg of isolated yeast mitochondria (Daun *et al.*, 1982) or an equal amount of canine pancreatic microsomal membrane (Promega) in import buffer (Kassenbrock *et al.*, 1993) in a 100-μl reaction at 25 °C for 20 min. Then membranes were washed through 20% sucrose cushion and reisolated. Protein integrated in membrane was analyzed by alkaline extraction of the membranes (Kassenbrock *et al.*, 1993). Protease pretreatment was done by incubating mitochondria with indicating concentration of protease K on ice for 30 min before sufficient inhibitor phenylmethylsulfonyl fluoride was added. Then, mitochondria was reisolated and resuspended in import buffer to start import reaction as described above.

RESULTS

In Vitro and in Vivo Expression of ISP6—The ISP6 gene encodes a small membrane protein of 61 amino acids that contains a membrane-spanning domain near its carboxyl terminus (Kassenbrock *et al.*, 1993). This yields an amphipathic protein that may be an important property for its function but makes it difficult for *in vitro* studies. In early attempts to generate *in vitro* forms of the protein for analysis of assembly and import, we observed that the small protein could only be translated in wheat germ extract but somehow not in reticulocyte lysate and that the resulting peptide was not soluble. It could be pelleted under the same conditions required for pelleting mitochondrial membranes and therefore was not suitable for biogenesis studies.

[1] The abbreviation used is: DHFR, dihydrofolate reductase.

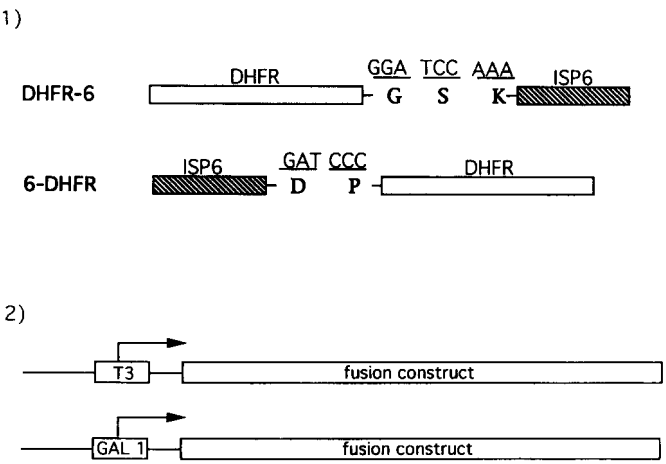

FIG. 1. **Map of the ISP6 fusion proteins constructed and used in this study.** *1)* DHFR-6 was constructed by joining ISP6 coding sequence with the carboxyl-terminal end of DHFR through an introduced *Bam*HI site. 6-DHFR was made by fusing the carboxyl end of ISP6 gene with the amino end of DHFR through a *Bam*HI site. *2)* Both of the constructs were placed under the control of bacterial T3 promoter for *in vitro* transcription and translation. These fusion constructs were also expressed *in vivo* under the control of GAL1 promoter in yeast plasmid pRS315.

In order to circumvent these problems and to yield a protein that could be monitored in import studies, gene fusions between a soluble protein, mouse DHFR, and ISP6 were constructed. Two different sets of constructions were prepared. First, DNA encoding the full-length ISP6 was fused in frame at the amino-terminal of the gene encoding mouse dihydrofolate reductase (6-DHFR). Second, the full-length ISP6 was fused at the carboxyl-terminal end of DHFR (DHFR-6). In each case (Fig. 1), these genes were placed in T3 promoter-based *in vitro* transcription-translation systems for the preparation of fusion proteins for *in vitro* studies. These same constructs were also placed in a yeast expression vector behind the Gal1 promoter for conditional expression of the gene product in yeast cells. *In vitro* translations of either the 6-DHFR or DHFR-6 gene products yielded proteins of the same size on SDS gels (data not shown).

In the first set of experiments, the gene fusion products were utilized to examine the association of ISP6 present at either the amino-terminal end or the carboxyl-terminal end of DHFR to different membrane fractions. Earlier studies had demonstrated that the mouse DHFR, which is a cytoplasmic protein in the cell, was unable to associate with or target to mitochondria (Hurt *et al.*, 1984). *In vitro* translated 6-DHFR or DHFR-6 were incubated with isolated yeast mitochondria and microsomes under standard conditions as shown in Fig. 2. Only the gene fusion product with ISP6 sequences located at the carboxyl-terminal end of the fusion protein associated strongly with mitochondria. When the level of gene fusion protein associated with mitochondria was quantitated, only 8% of the gene fusion product with ISP6 at the amino-terminal end (6-DHFR) remained with mitochondria, whereas 33% of the input DHFR-6 gene fusion product cofractionated with mitochondria under these same conditions. Both fusion proteins had the ability to associate with the isolated microsomal membranes, but at a reduced level compared with their association with mitochondria. These data support the earlier observation (Kassenbrock *et al.*, 1993) that the ISP6 protein is associated with the outer mitochondrial membrane by its carboxyl-terminal membrane anchor. In addition, the data also suggest that the insertion of the small protein into the membrane apparently is via its carboxyl-terminal end first.

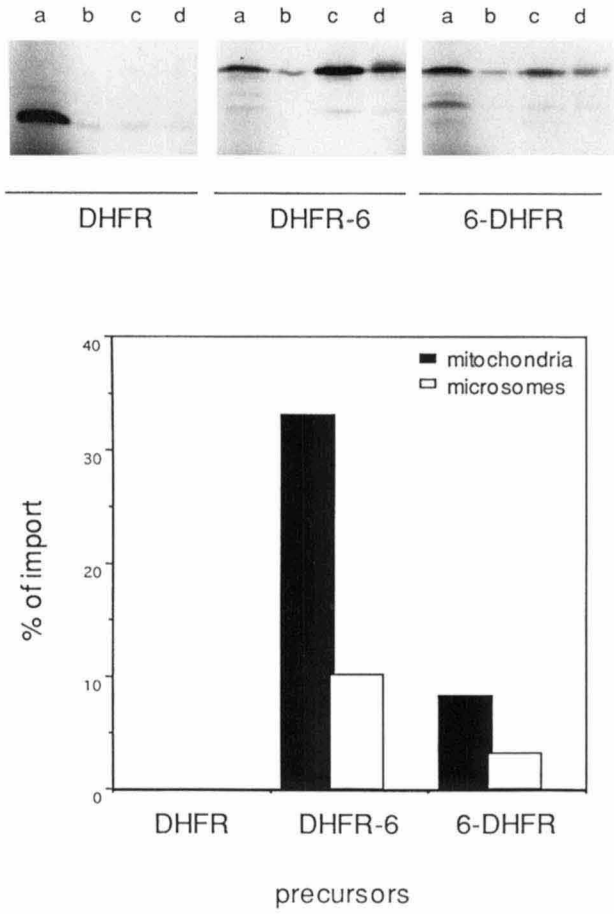

FIG. 2. ***In vitro* insertion of ISP6-DHFR fusion proteins into membranes.** *In vitro* translated DHFR-6, 6-DHFR, as well as DHFR proteins were incubated without membrane (*b*), with 50 μg isolated mitochondria (*c*), or with equivalent amount of isolated microsomal membrane (*d*) in import buffer for 20 min at 25 °C. The portion of input proteins resistant to Na_2CO_3 extraction after import was loaded on SDS-PAGE and autoradiographed as shown here. 20% of total input proteins is shown in *a*.

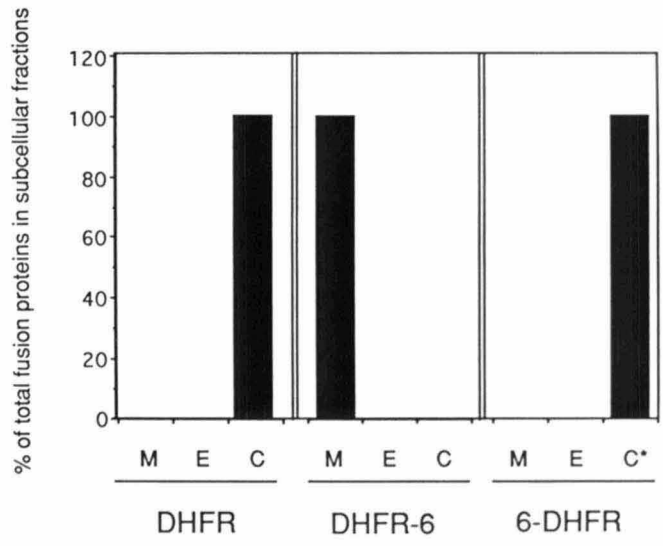

FIG. 3. ***In vivo* subcellular localization of ISP6-DHFR fusion proteins.** Yeast cells expressing indicating fusion proteins as well as DHFR were fractionated into mitochondria (*M*), crude microsomes (*E*), and cytosolic fraction (*C*). The level of the proteins associated with each fraction was examined by Western blot with anti-DHFR antibody and then quantitated. *, 6-DHFR was found degraded into a smaller fragment, which remains exclusively in cytosol.

To examine the localization of the ISP6 protein *in vivo*, yeast shuttle vectors containing the gene fusions under the control of the Gal 1 promoter were introduced into wild-type strains, and the fate of the DHFR fusion protein was monitored. Following growth of yeast cells in the appropriate selective media, cell fractions were prepared for the quantitation of DHFR in different subcellular fractions. For a control in this study, the DHFR protein by itself was also expressed and monitored for its distribution. As shown in Fig. 3, all of the DHFR fusion expressed in yeast, which harbor the ISP6 sequences at the carboxyl-terminal end, was efficiently targeted and localized in the mitochondrial fraction. In contrast, like the DHFR protein itself, the DHFR fusion harboring ISP6 sequences at the amino-terminal end was localized exclusively to soluble fractions. We also observed that the incorrectly targeted 6-DHFR fusion product in these studies was more labile to degradation than the DHFR or DHFR-6 expressed under identical conditions. As a control for these *in vivo* studies, we also pulse labeled these proteins to confirm that the same level of DHFR-6 and 6-DHFR proteins were synthesized under these conditions (data not shown).

DHFR-6 But Not 6-DHFR Is Correctly Targeted in Vivo—The *in vivo* distribution described above strongly implies, like the *in vitro* data, that the carboxyl-terminal localized ISP6 sequences are effective in targeting DHFR reporter to the mitochondrial fraction. In order to gain additional support for the correct targeting of this protein, we exploited the earlier observation that the isp42–3 allele, which is conditionally defective in protein import at 35 °C, can be rescued by overexpression of the wild-type ISP6 protein (Kassenbrock *et al.*, 1993). In each case, the two ISP6-DHFR gene fusions and DHFR itself were transformed into a yeast strain harboring the isp42–3 allele. When the resulting transformants were left to grow at nonpermissive temperature, only the strain expressing the DHFR fusion with ISP6 sequences at the carboxyl-terminal end was effective in supporting growth at high temperature (Fig. 4). These data provide additional support that the correct targeting of the DHFR gene fusion occurred even though it contained a DHFR domain at its amino terminus.

If oriented in the same manner as the ISP6 wild-type protein, DHFR-6 fusion should localize in such a way to the mitochondrial surface that it places the soluble DHFR domain on the cytoplasmic face of the outer mitochondrial membrane. To confirm the orientation of the fusion protein, the correctly targeted DHFR-6 fusion products associated with the mitochondria were further examined using proteolysis. As shown in Fig. 5, this analysis revealed that the DHFR domain present on the mitochondrial surface was readily accessible to added protease under the conditions in which cytochrome b_2, a marker protein localized in the intermembrane space, was not accessible. This construct, therefore, renders the DHFR-6 gene fusion product a member of the carboxyl-terminal anchored protein family.

Mitochondrial Delivery in the Absence of Outer Membrane Receptors—The carboxyl-terminal targeted protein, ISP6, is a member of a new class of proteins in the cell, which clearly must be completed as a nascent protein before being properly localized (Kutay *et al.*, 1993). In order to understand the extent to which different mitochondrial receptors operate in the delivery of ISP6, two experiments were performed. In the first, we exploited the genetic deletions of two mitochondrial outer membrane receptors, *i.e.* MAS20 and MAS70, that had been previously characterized (Ramage *et al.*, 1993). In each case, we prepared mitochondria from yeast strain lacking one of the two receptors and incubated the labeled fusion proteins with these mitochondria (Fig. 6*A*). This experiment demonstrates that

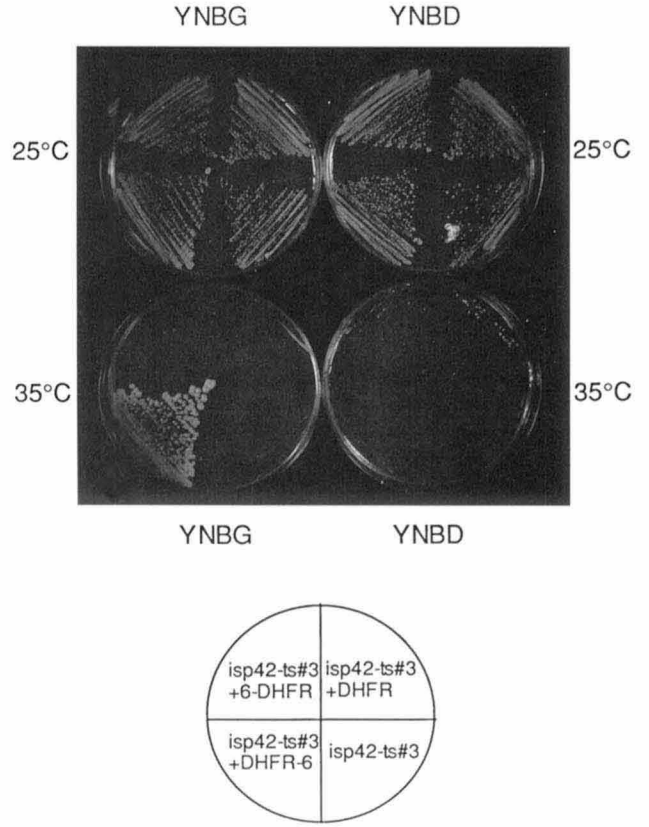

FIG. 4. **Suppressor function of ISP6-DHFR fusion proteins.** Temperature-sensitive mutant isp42–3 cells transformed with indicating fusion proteins were streaked on plates with inducing (*YNBG*) or noninducing (*YNBD*) carbon source and incubated at permissive (25 °C) or nonpermissive (35 °C) temperature for 10 days.

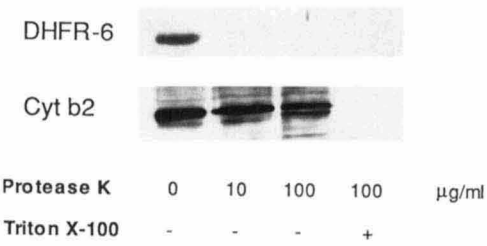

FIG. 5. **Orientation of *in vivo* targeted DHFR-6 on mitochondria membrane.** Mitochondria (100 μg) isolated from yeast cells expressing DHFR-6 protein were subjected to digestion by externally added protease K for 30 min on ice. After digestion, the levels of DHFR-6 and marker protein cytochrome b_2 were examined by Western blot. As a control, in one reaction the mitochondria membrane was first solubilized with 1% Triton X-100 before protease K was added.

there was no difference between wild-type mitochondria and mitochondria depleted of either protein receptor. However, as expected, the import of F1α subunit precursor was greatly impaired in mitochondria isolated from ΔMAS20 strain. We transformed the mutant strains containing deletions in either MAS20 or MAS70 with the fusion construct and determined the extent to which the DHFR-6 was localized. We observed that the localization of the DHFR-6 under these conditions was identical in both strains (data not shown) and the same as wild type. Thus, the data above suggest that neither MAS20 or MAS70 plays a role in the sorting of ISP6.

In order to further characterize this targeting, we determined if proteins exposed on the mitochondrial surface might be necessary for binding and insertion of ISP6 into isolated mitochondria. In this study, mitochondria were pretreated with protease prior to the import reaction to determine the consequences on import. As shown in Fig. 6*B*, we observed that mild digestion of isolated mitochondria with proteinase K yielded mitochondria that were unable to import presequence containing precursors such as the $F_1\alpha$ subunit. Under these conditions and even at concentrations of the protease well above that required to inhibit import of F_1 ATPase precursor (*e.g.* 200 μg/ml protease K), the binding and apparent insertion of DHFR-6 remained unchanged. To access the integrity of ISP42 under these conditions, we examined ISP42 by immunoblotting (Fig. 6*B*). This protein was effectively proteolyzed even at 10 μg/ml, and a smaller fragment was generated that was also gradually digested at higher protease levels. These data indicate that the targeting mechanism that operates for the carboxyl-terminal insertion of the ISP6 protein is by a mechanism not previously characterized.

ISP42 Is Not Required for Delivery of ISP6—In earlier studies, it was proposed that ISP6 might operate *in vivo* to stabilize the ISP42 protein in its assembly and or dynamics for correct precursor translocation activity. One possibility for the targeting of ISP6 might be that its assembly with ISP42 in the outer membrane was acting to promote the specific delivery in some manner. To test this model, antibodies against either carboxyl-terminal end, amino-terminal end, and full length of ISP42 were prebound to mitochondria to block association of the DHFR-6 fusion protein. Under these conditions, the insertion was not affected (data not shown) in any detectable manner.

In earlier studies, we have determined that the *in vitro* import of proteins into an ISP42-ts mutant can be efficiently blocked at higher temperature under conditions in which similar import into the wild-type mitochondria remains unchanged (Kassenbrock *et al.*, 1993). To determine if the absence of functional ISP42 might influence the association of ISP6 with mitochondrial membrane, we exploited the lability of the isp42–3 mutant. In this experiment, mitochondria preparations from ISP42 wild type and the isp42–3 temperature-sensitive mutant were held at the nonpermissive temperature for 10 min prior to the initiation of the *in vitro* import reaction. Under these conditions, we observe that the import of the DHFR-6 protein was not affected in the isp42-ts mutant when compared with the wild type (Fig. 7).

On the other hand, when the integrity of ISP42 in the protease pretreated mitochondria was examined (Fig. 6*B*), we found that DHFR-6 fusion protein inserted efficiently into mitochondrial outer membrane even after more than 90% of ISP42 was proteolyzed (protease K concentration at 200 μg/ml). Thus, all of the data suggest that ISP42 unlikely functions as a targeting partner to DHFR-6 fusion protein.

DISCUSSION

In this paper, we have examined the biogenesis of ISP6 and have observed some unusual features of its targeting. Introduction of DHFR domain at the carboxyl-terminal end of ISP6 completely abolished the ability of the resulting 6-DHFR to target and function appropriately *in vivo*. On the other hand, efficient insertion of DHFR-6 to mitochondrial outer membrane does not require the involvement of proteinaceous receptors, such as MAS20 and MAS70. ISP42, which forms a multiprotein complex with ISP6 on mitochondria outer membrane, does not likely play a role in targeting of ISP6.

Based on the observations described here, ISP6 appears to belong to a new membrane protein class that possesses a hydrophobic segment near the carboxyl terminus that orients it with its amino terminus in the cytosol (Kutay *et al.*, 1994). Among the two forms of ISP6-DHFR fusion proteins, only DHFR-6 with its native carboxyl-terminal end can target itself to its correct destination and maintain its suppresser function for the isp42–3 mutant. However, the targeting information

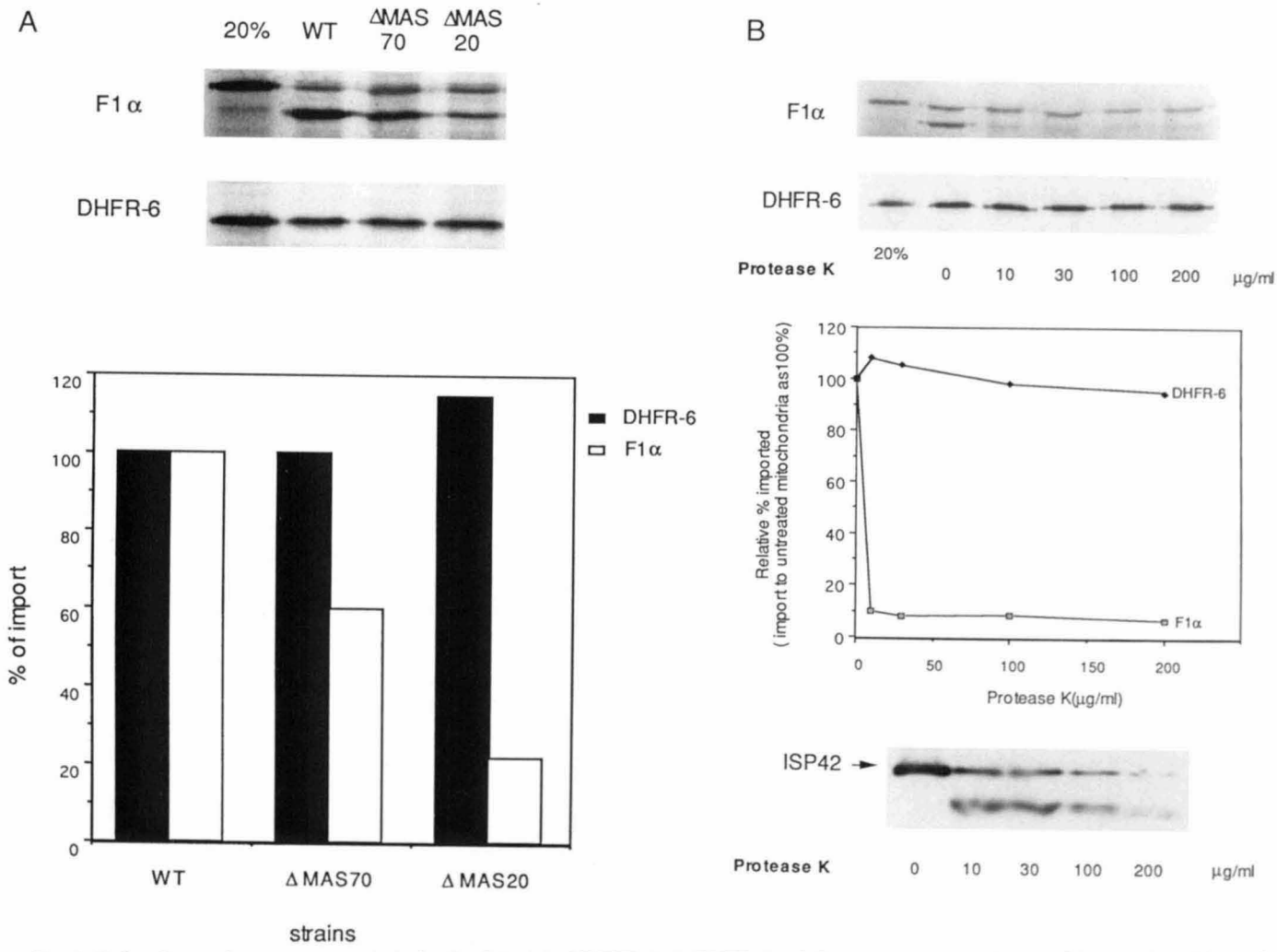

FIG. 6. **Role of proteinaceous receptors in the import of DHFR-6.** *A*, DHFR-6 and F1α precursor were imported *in vitro* into 50 μg of mitochondria isolated from wild-type W303 strain and mutant strains deficient of receptor protein MAS70 or MAS20, respectively. *B*, isolated wild-type mitochondria (50 μg) were preincubated with indicating concentrations of protease K before the treated mitochondria were reisolated and mixed with translated DHFR-6 or pre-F1α in import buffer and incubated at 25 °C for 20 min. The level of ISP42 associated with the predigested mitochondria was examined by Western blot.

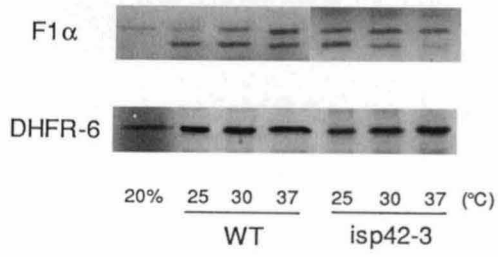

FIG. 7. **Role of ISP42 in the import of DHFR-6.** Mitochondria isolated from wild-type strain (*WT*) or ts mutant isp42–3 (50 μg each) were preincubated at different temperatures for 10 min before labeled DHFR-6 or F1α precursor was added and incubated for another 20 min at the same temperature.

seems to be disrupted in the construct 6-DHFR, in which the DHFR domain was placed at the carboxyl terminus of ISP6. Therefore, ISP6 appears to rely on sequences that must be correctly presented near the carboxyl-terminal end of the protein. This is very similar to the carboxyl-terminal anchored proteins, which are found directed to their subcellular destinations by the sequences either adjacent to or inside the carboxyl-terminal anchor (Kutay *et al.*, 1993; Mitoma and Ito, 1992; Nguyen *et al.*, 1993).

Control of the targeting specificity for carboxyl-terminal anchored proteins is of special importance since their hydrophobic tails have the potential to interact with different membranes. It has been shown that some of the carboxyl-terminal anchored proteins can insert into any membrane and even liposomes spontaneously *in vitro* (Mitoma and Ito, 1992; Janiak *et al.*, 1994). The DHFR-6 fusion was also able to associate *in vitro* with membranes other than mitochondrial membrane, *e.g.* microsomal membranes, while at a reduced level when compared with its association with mitochondria. The fact that *in vivo* this protein targeted exclusively to mitochondria indicates that certain mechanisms are operating to ensure the specific delivery.

However, import receptors previously described for entry of most mitochondrial precursors are apparently not required for entry of ISP6. The localization of ISP6 to mitochondrial outer membrane has been found unaffected *in vivo* and *in vitro* in yeast cells depleted of either MAS20 or MAS70 receptors. Furthermore, ISP6 can be effectively imported *in vitro* into mitochondrial outer membrane in which functional surface receptors have been eliminated. This form of receptor-independent targeting is very unusual and has been described for only a few mitochondrial precursor proteins (Hartl and Neupert, 1990). Among them, MOM19, the counterpart of MAS20 in *Neurospora crassa*, is the only example of such a protein on the outer membrane (Schneider *et al.*, 1991). The biogenesis of Bcl-2, another carboxyl-terminal anchored protein, has been shown to associate with mitochondrial outer membrane via a mechanism

yet to be confirmed (Nguyen *et al.*, 1993; Janiak *et al.*, 1994).

The selective targeting and assembly of the components of any translocation machinery to its correct organelle membrane is an essential prerequisite to maintain the specific organization of a eukaryotic cell. For the ISP6 protein, it is not clear what is responsible for the specificity of this process since the common receptors operating for other components are not involved. Closely associated with ISP6 in the translocation complex, the ISP42 gene product was then speculated as a potential receptor to assist insertion of ISP6 through their assembly. In *N. crassa* mitochondria, MOM38, the homologue of ISP42 has been proposed to play such a role in the targeting of the master receptor MOM19 (Schneider *et al.*, 1991). However, three observations described here show that ISP42 is probably not the receptor for the specific insertion of ISP6. 1) Prebound antibodies against ISP42 to mitochondria surface did not block the insertion of DHFR-6. 2) Import of DHFR-6 remains efficient in the mitochondria, which lost the translocation activity of ISP42. 3) Proteolysis of ISP42 did not inhibit the import of DHFR-6. Although ISP42 may not be the receptor that helps docking and inserting ISP6, the mitochondrial outer membrane may have other proteins that can be recognized by ISP6 and play such a role. The identification of the specific components residing on the outer membrane as well as the sequence requirements for the targeting of ISP6 are currently under investigation.

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